(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) Publication number: 0 317 804 A2

(12) EUROPEAN PATENT APPLICATION

(21) Application number: 88118299.2

(22) Date of filing: 03.11.88

(51) Int. Cl.4: C07K 7/10 , G01N 33/68 , A61K 37/02

(30) Priority: 24.11.87 US 124801

(43) Date of publication of application: 31.05.89 Bulletin 89/22

(84) Designated Contracting States: AT BE CH DE ES FR GB IT LI NL

(71) Applicant: ABBOTT LABORATORIES
Abbott Park Illinois 60064(US)

(72) Inventor: Sarin, Virender K.
516 Fairlawn Avenue
Libertyville Illinois 60048(US)
Inventor: Knigge, Kevin M.
1083 Lawson Blvd.
Gurnee Illinois 60031(US)

(74) Representative: Modiano, Guido et al
MODIANO, JOSIF, PISANTY & STAUB
Modiano & Associati Baaderstrasse 3
D-8000 München 5(DE)

(54) HIV peptides and methods for detection of HIV.

(57) Novel synthetic peptides which substantially replicate various sequences of gp160 envelope protein of the human immunodeficiency virus (HIV) are disclosed. Also disclosed are methods for detection of antibodies to HIV in biological samples using the gp160 peptides.

EP 0 317 804 A2

HIV PEPTIDES AND METHODS FOR DETECTION OF HIV

BACKGROUND OF THE INVENTION

A retrovirus termed human immunodeficiency virus (HIV) is now known to be the etiologic agent in acquired immune deficiency syndrome (AIDS). Various isolates of this virus have been termed lymphadenopathy-associated virus (LAV), human T-cell lymphotropic virus type-III (HTLV-III) or AIDS-associated retrovirus (ARV). Although the modes of transmission for HIV are not completely understood, the most common forms of transmission of the HIV virus are through sexual contact, use of contaminated intravenous equipment and transfusions with contaminated blood products. Testing for HIV has become extremely important in diagnosing exposure to the virus and particularly for protecting blood products from contamination.

To detect exposure to HIV, levels of HIV antibodies (anti-HIV) are measured in serum, plasma, saliva or other biological samples of HIV patients or individuals at risk for AIDS. In the first generation of tests for HIV antibodies, inactivated crude or purified viral protein from lysates of HIV-infected cells are used to coat a solid phase. The lysate-coated solid phase is incubated with a biological sample suspected of containing anti-HIV, washed and then anti-human antibody tagged with a detectable label is added to the solid phase. The label, which may be an enzyme, radioisotope or fluorescent molecule is measured to determine presence of HIV antibodies in the sample.

The problem with using viral lysates as a source of HIV antigens is that the infectious nature of the HIV virus makes manufacturing the virus potentially hazardous. Also, HIV viral lysates may contain impurities which interfere with testing for HIV antibodies. Therefore, alternate sources of HIV antigens are needed which are safe and noninfectious. There is also a need for HIV antigens which are well-defined and do not cross-react with other non-HIV antibodies and other interfering materials contained in the sample to be tested.

Studies have shown that HIV antibodies to gp160 and gp120 are found in human saliva of asymptomatic, AIDS-related complex and AIDS patients. Archibald, et al., Blood, 67:831-834 (1986). These salivary antibodies represent mostly IgA immune response.

Various isolates of HIV have been cloned and their genetic structures established. Nucleotide and deduced amino acid sequences of the various regions of the HIV genome are published. However, the published sequences do not provide any information as to which portions of the HIV molecule, when synthesized as peptides, would have antigenic or immunogenic properties similar to the corresponding region of naturally-occurring HIV proteins.

The envelope (env) region of the HIV gene encodes an approximately 856 residue precursor protein with various potential glycosylation sites. The precursor glycoprotein corresponds to a molecular weight of 160,000 daltons (gp160) and is processed at the -Lys Arg- pair to yield an N-terminal protein of 480 amino acids (gp120) and a 345 amino acid protein (gp41).

It has been demonstrated by radioimmune precipitation followed by polyacrylamide gel electrophoresis (RIP-PAGE) as well as by enzyme-linked immunosorbent assay (ELISA) that sera from AIDS and AIDS-related complex (ARC) patients react with env and gag gene encoded proteins of HIV. These proteins include but are not limited to gp160 gp120, gp41, p55, p36, p24, p18 and p12.

In European Patent Application No. 227,169, Berman et al. disclose several polypeptides mimicking amino acid sequences of AIDS-related viral proteins. An immunochemical reagent is made by combination of two or more of the synthetic polypeptide sequences selected from the following group: a polypeptide sequence mimicking at least one antigenic determinant of the gag antigen of HIV, a polypeptide sequence mimicking at least one antigenic determinant of the glycoprotein gp120 of HIV and a polypeptide sequence mimicking at least one antigenic determinant of the glycoprotein gp41 of HIV. Berman et al. state that the polypeptide sequences disclosed should be substantially free of the naturally occurring gag, gp120 and gp41 proteins of HIV even though these reagents may optionally contain naturally occurring HIV proteins. The Berman et al. disclosure provides no indication as to which HIV antigen or epitope would be important for detecting salivary antibodies.

In Wong-Staal, et al., U.S. Pat. Applic. Ser. No. 779,431, a 15 amino acid long peptide from gp120 is described. This peptide is useful as an immunogen in the production of monoclonal antibodies and in an ELISA assay.

In Proc. Nat'l Acad. Sci. USA, 84:2479-2483 (1987), Palker, et al. disclose a synthetic peptide derived

BNSDOCID: <EP 0317804A2>

from the COOH-terminal region of gp120 containing 15 amino acids. This relatively short peptide was used to evaluate reactivity of HIV-positive patients' antibodies and as an immunoadsorbent to evaluate functional importance of human antibody response to the COOH terminus of gp120.

In Cosand, U.S. Patent No. 4,629,783, peptides having sequences mimicking short regions of gp120 gp41 and p24 are disclosed. Those peptides, which can be used for detection of anti-HIV in blood screening procedures, mimic proteins encoded by the gag or env regions of the viral genome. Preferably, the peptides disclosed by Cosand contain fewer than 25 amino acids.

SUMMARY OF THE INVENTION

We have developed novel gp160 peptides which can be used for detecting HIV antibodies in biological samples, for producing HIV antibodies and for vaccines. These peptides substantially mimic regions of gp160 or its gp120 fragment and provide noninfectious and pure sources of HIV antigens.

First, a short peptide (Peptide I) corresponding to amino acids 490-517 of gp120 encoded in the region between base pairs (bp) 7221-7305 [Sanchez-Pescador, et al., Science, 227:484-492 (1985)] was synthesized for testing immunogenicity/antigenicity.This peptide sequence is shown below:

(I) Y-Tyr-Lys-Tyr-Lys-Val-Ile-Lys-Ile-Glu-Pro-Leu-Gly-Ile Ala-Pro-Thr-Lys-Ala-Lys-Arg-Arg-Val-Val-Gln-Arg-Glu-Lys-Arg-X,

wherein Y corresponds to -H, a blocking group, an amino acid, peptide, protein or any linker and X may be -OH, $-NH_2$, $-NR_1R_2$ (wherein R corresponds to an alkyl group), a peptide, protein or any linker.

The amino acid sequence of Peptide II corresponds to amino acids 482-517 of gp120 encoded in the region between bp 7197-7305 [Sanchez-Pescador, et al., supra]. The amino acid sequence for Peptide II is shown below:

(II) Y-Arg-Asp-Asn-Trp-Arg-Ser-Glu-Leu-Tyr-Lys-Tyr-Lys-Val-Ile-Lys-Ile-Glu-Pro-Leu-Gly-Ile-Ala Pro-Thr-Lys-Ala-Lys-Arg-Arg-Val-Val-Gln-Arg-Glu-Lys-Arg-X,

wherein Y and X are the same as described above for Peptide I.

Peptide III extends from amino acids 455 to 511 of gp120 encoded in the region between bp 7165-7335 [Muesing, et al., Nature, 313:450-458 (1985)]. The amino acid sequence of this peptide is shown below:

(III) Y-Thr-Arg-Asp-Gly-Gly-Asn-Ser-Asn-Glu-Ser-Glu-Ile-Phe-Arg-Pro-Gly-Gly-Gly-Asp-Met-Arg-Asp-Asn-Trp-Arg-Ser-Glu-Leu-Tyr-Lys-Tyr-Lys-Val-Val-Lys-Ile Glu-Pro-Leu-Gly-Val-Ala-Pro-Thr-Lys-Ala-Lys-Arg-Arg-Val-Val-Gln-Arg-Glu-Lys-Arg-X,

wherein Y and X are as explained for Peptide I.

Peptide IV encompasses the C-terminus of gp120 and N-terminus of gp41 and has an amino acid sequence from amino acids 480 to 529 encoded by the region extending from bp 7240-7389 [Muesing, et al., supra]. The Peptide IV sequence is shown below:

(IV) Y-Arg-Ser-Glu-Leu-Tyr-Lys-Tyr-Lys-Val-Val-Lys-Ile-Glu-Pro Leu-Gly-Val-Ala-Pro-Thr-Lys-Ala-Lys-Arg-Arg-Val-Val-Gln-Arg-Glu-Lys-Arg-Ala-Val-Gly-Ile-Gly-Ala-Leu-Phe-Leu-Gly-Phe-Leu-Gly-Ala-Ala-Gly-Ser-Thr-X,

wherein Y and X are as explained above.

These peptides can be used as sources of HIV antigens alone or in combination or may be linked to larger carrier molecules. It is preferred that one or more of the Peptides I-IV be used in combination with other antigens and epitopes, particularly other HIV antigens such as p24 and gp41.

The peptides of the invention can be synthesized by a number of methods including synthesis in solution or by solid phase peptide methodology using stepwise or fragment coupling protocols. They can also be synthesized enzymatically or made as fused proteins or peptides by recombinant DNA methodology. Regions of genes coding for these peptides may also be synthesized, cloned and expressed using recombinant DNA technology, and the sequences may be subcloned and expressed in suitable expression systems such as E.coli, yeast or mammalian cells. The peptides described herein may be combined with

BNSDOCID: <EP 0317804A2>

other epitopes or antigens of HIV, with or without carrier molecules, and expressed by recombinant DNA methods or made by synthetic methods as fusion proteins. Substitution, deletion or addition analogs of the peptides of the invention can also be made by methods well known to those skilled in the art.

The peptides corresponding to the various examples herein can be used alone, in combination with one another or in combination with other antigens of interest. For example, various of these peptides can be used as physical mixtures or chemically coupled to each other with or without spacer molecules. It is also possible to couple peptides chemically or physically with carrier peptides, proteins or supports. These peptides can be used with other HIV antigens or epitopes such as gp41, gp120, p55, p24 and others. The peptides, particularly Peptide III, can be coupled to carrier molecules like thyroglobulin or BSA and used as antigens alone or in combination with other epitopes or antigens.

DETAILED DESCRIPTION OF THE INVENTION

The following examples illustrate methods of making the peptides, as well as methods of using the peptides as sources for pure, well-defined, noninfectious HIV antigens.

Example 1

This example illustrates the synthesis of Peptide I on a resin support by stepwise solid phase synthesis starting with the carboxy-terminal residue. A procedure such as the procedure described in Barany and Merrifield, The Peptides, 2:1984, Gross, E., and Meinehofer, J., Eds., Academic Press, New York, N.Y. (1980) can be used for this synthesis. A Boc-L-Arg(Tos)-OCH_2-Pam resin was transferred to a reaction vessel of an Applied Biosystems Synthesizer, Model 430A, available from Applied Biosystems, Foster City, California. Protected amino acids were coupled in a stepwise manner to the resin support by preformed symmetric anhydride chemistry, except in the cases of arginine, asparagine and glutamine addition where the DCC/HOBT protocol described by Konig and Geiger, Chem. Ber., 103:788 798 (1970) was employed. All amino-terminal residues were protected by t-butyloxy carbonyl (t/BOC-linkage) and side chains of various amino acid residues were protected by the following groups: Arg, Tos; Lys, 2-ClZ; Glu, OBZ1; Thr, Bz1; Tyr, 2-BrZ.

The fully protected peptide-resin (0.7 g) was allowed to swell in methylene chloride (CH_2Cl_2) for 5 minutes. The N^{α}-Boc protecting groups were removed using 60% trifluoroacetic acid (TFA/CH_2Cl_2) deprotection, CH_2Cl_2 washes, 10% N,N diisopropylethylamine (DIEA/CH_2Cl_2) neutralization and finally washing with CH_2Cl_2 again. The resin was dried in vacuo. The peptide resin so obtained was treated with 9 ml of anhydrous hydrofluoric acid (HF) to which 1 ml of p-cresol had been added, for 60 minutes at 0° C. The HF was distilled off in vacuo at 0° C. The cleaved free peptide and resin were washed 3 times with 15 ml aliquots of diethyl ether, and the peptide was extracted by means of 3 extractions with 15 ml of 40% aqueous acetic acid. The aqueous extracts were combined and washed three times with 10 ml aliquots of diethyl ether, whereupon the aqueous layer was lyophilized to provide the crude peptide for purification. The polypeptide was purified by reversed-phase high performance liquid chromotography (HPLC) on C_4 columns employing gradients of 0.1% TFA/water (A) and 100% acetonitrile (B) as the solvent systems at a 1 ml/min. flow rate for the analytical (Vydac-214-TP54, Vydac Separation Group, Hesperia, California) or 3 ml/mm flow rate for the semi-preparative (Vydac-214-TP510) columns. The gradient used was:

	1 min		20 min		1 min	
20%B	→	20%B	→	25%B	→	20%B

The polypeptide elution from the HPLC column was monitored at 222 nm and 280 nm. The composition of the polypeptide was confirmed by hydrolysis in 6 N hydrochloric acid (HCl)/0.3% phenol at 150° C for 2 hours in vacuo and subsequently analyzed on a Beckman 6300 amino acid analyzer with a SICA 7000 A integration available from Beckman Instruments, LaBrea, California.

Peptides II, III and IV were synthesized in a manner similar to the one described above. Methionine was used in the sulfoxide form, trytophan was protected by the formyl group (CHO); Ser, Bzl; Asp, OBZL. For

BNSDOCID: <EP 0317804A2>

peptide-resins containing methionine sulfoxide and formyl-tryptophan, peptides were deprotected and cleaved off the resin using "low high" HF protocols as described by Tam, et al., J. Am. Chem. Soc., 105:6442-6455 (1983). Desired peptides can also be synthesized using unprotected methionine and tryptophan with the appropriate uses of scavengers during deprotection and cleavage. All peptides were purified using reversed phase C_4 HPLC wih 0.1% aqueous TFA and a 100% acetonitrile gradient system.

Peptides I, II, III and IV may be conjugated to larger carrier molecules such as bovine serum albumin (BSA), keyhole limpet hemocyanin (KLH) or thyroglobulin using water soluble carbodiimide or maleimido-benzoyl-N-hydroxysuccinimide (MBS) as described in Liu, et al., Biochem., 18:690-697 (1979), and Kitagawa, et al., J. Biochem., 92:585-590 (1982). These conjugates can then be used to raise sequence specific antibodies.

Example 2

This example demonstrates a method of detecting HIV antibodies in biological samples using the peptides described in Example 1. Biological samples which can be tested by the methods described herein include blood preparations such as serum or plasma, urine and saliva. The peptides of the invention are particularly useful in a saliva test for the presence of IgA, IgG or IgM antibodies to HIV, especially when the peptides are combined with other HIV antigens.

Peptides I and III were tested side-by-side with recombinant DNA-derived HIV p24 and HIV gp41 to determine their usefulness as sources of HIV antigens in a method for detecting HIV antibodies in biological samples.

Four antigen solutions were prepared in 50 mM sodium carbonate, pH 9.5 as follows: Peptide I, 10 ug/ml; Peptide III, 6.25 ug/ml; recombinant DNA-derived HIV p24 protein, 1.64 ug/ml; and recombinant DNA-derived HIV gp41 protein, 125 units/ml. The p24 and gp41 proteins were made by methods described in copending U.S. Patent Application Serial No. 020,287, filed Feb. 27, 1987, which is incorporated by reference herein and assigned to the same assignee as that of the present invention.

One hundred microliters (100 ul) of each antigen solution were added to wells of a polystyrene microtiter plate available from Dynatech Laboratories, Alexandria, Virginia. Other solid phases which may be utilized in the methods described herein include beads, paper strips, microparticles, nitrocellulose membranes, polystyrene tubes or other suitable plastic or paper supports. The solution and the plate were incubated for 1 hour at room temperature after which the solution was removed and the plate washed five times with distilled water. Two hundred and fifty microliters of an overcoat solution consisting of 10% bovine serum albumin, 3% sucrose and 0.05% Tween 20 in phosphate buffered saline (PBS) (0.01 M KH_2P0_4; 0.15 M NaCl: pH 7.2) were added to the wells. Following a 30 minute incubation, the overcoat solution was removed and the plate washed five times with distilled water. Coated plates were stored at 2-8° C for subsequent use.

The coated plates were used in an assay for detecting anti-HIV as follows: one hundred microliters of a serum sample diluted 1:800 or a saliva sample diluted 1:10 in a diluent consisting of 10% bovine serum albumin and 2% Tween 20 in phosphate buffered saline (0.01 M KH_2P0_4; 0.15 M NaCl: pH 7.2) were added to wells of the coated microtiter plates. After a 1 hour incubation at room temperature, the sample was removed and the wells washed five times with distilled water. One hundred microliters of an antibody-enzyme conjugate (alkaline phosphatase:goat anti-human IgG or alkaline phosphatase:goat anti-human IgA) were added to the wells. The antibody-enzyme conjugate can also be an IgM antibody conjugate if IgM antibody is being detected.The antibody-enzyme conjugates are made as described in Engvall, et al., Biochim. Biophys. Acta, 251:427-434 (1971); Korn et al., J. Mol. Biol., 65:525-529 (1972); and Avrameas and Ternynck, Immunochemistry, 6:53-66 (1969). Following another 1 hour incubation at room temperature, the conjugate was removed and the wells washed five times with distilled water. One hundred microliters of a p-nitrophenylphosphate substrate solution were added to the wells and incubated at room temperature for 30 minutes. One hundred microliters of 2N NaOH were added to the wells to stop the reaction. Absorbance values of the wells were read at 405 nm. A positive result was determined at a cutoff value of 0.200 O.D. or greater, which was established based on the results generated from 100 negative and 50 positive paired serum and saliva samples. The results are set forth in Tables 1, 2 and 3.

The results indicate that all confirmed seropositive samples tested contained serum IgG to gp41, Peptide I and Peptide III, but the serum reactivity to p24 varied. In saliva, the IgG reactivity in seropositive samples varied for all antigens tested. Although the salivary IgA reactivity varied for p24 and gp41, all seropositive samples contained salivary IgA to Peptide I and Peptide III. For the confirmed negative

BNSDOCID: <EP 0317804A2>

samples, no reactivity was detected in either serum or saliva for any of the antigens tested. In general, the reactivity of Peptide III was greater than that of Peptide I.

These results demonstrate that Peptide I and Peptide III are specifically reactive with antibody to HIV. In addition, the results indicate that at least a portion of gp120 must be present when testing for antibodies to HIV in saliva samples.

Table 1

Patient Diagnosis	Confirmed Seropos.	Microtiter Serum IgG			
		p24	qp41	Peptide I	Peptide III
AIDS	Yes	0.089	>3.0	1.734	>3.0
AIDS	Yes	0.034	2.009	0.981	1.730
AIDS	Yes	0.003	>3.0	1.836	2.009
AIDS	Yes	0.006	2.105	0.301	0.873
AIDS	Yes	0.049	2.980	2.437	>3.0
AIDS	Yes	0.097	>3.0	>3.0	>3.0
AIDS	Yes	0.102	2.874	1.920	2.941
AIDS	Yes	0.031	2.541	1.337	2.226
ARC	Yes	0.198	>3.0	2.011	>3.0
ARC	Yes	0.157	>3.0	1.990	2.798
Asymptomatic	Yes	2.983	>3.0	1.933	2.322
Asymptomatic	Yes	>3.0	>3.0	>3.0	>3.0
Asymptomatic	Yes	0.895	>3.0	>3.0	>3.0
Asymptomatic	Yes	>3.0	>3.0	>3.0	>3.0
Asymptomatic	Yes	1.971	>3.0	2.909	>3.0
Hemophiliac	Yes	0.301	2.179	ND	1.899
Hemophiliac	Yes	1.127	2.357	ND	0.207
Hemophiliac	Yes	1.193	1.715	ND	1.119
Hemophiliac	Yes	0.702	2.770	ND	2.248
Hemophiliac	Yes	>3.0	>3.0	ND	>3.0
Hemophiliac	Yes	0.077	2.552	ND	0.558
Hemophiliac	No	0.051	0.086	ND	0.003
Hemophiliac	No	0.006	0.029	ND	0.079
Hemophiliac	No	0.018	0.068	ND	0.044
High Risk	No	0.005	0.076	ND	0.101
High Risk	No	0.018	0.006	ND	0.022
Healthy Hetero.	No	0.000	0.114	0.043	0.097
Healthy Hetero.	No	0.003	0.093	0.055	0.081
Healthy Hetero.	No	0.019	0.008	0.092	0.005
Healthy Hetero.	No	0.058	0.055	0.003	0.037

BNSDOCID: <EP 0317804A2>

Table 2

Patient Diagnosis	Confirmed Seropos.	Microtiter Saliva IgG			
		p24	qp41	Peptide I	Peptide III
AIDS	Yes	0.044	0.971	0.204	0.568
AIDS	Yes	0.013	0.111	0.123	0.228
AIDS	Yes	0.033	0.758	0.229	0.318
AIDS	Yes	0.022	0.014	0.041	0.059
AIDS	Yes	0.005	0.296	0.476	0.935
AIDS	Yes	0.041	>3.0	2.037	>3.0
AIDS	Yes	0.027	0.246	0.398	0.837
AIDS	Yes	0.035	0.216	0.271	0.440
ARC	Yes	0.009	1.230	0.311	1.773
ARC	Yes	0.036	0.931	0.292	0.909
Asymptomatic	Yes	1.852	2.707	0.279	0.532
Asymptomatic	Yes	2.190	>3.0	>3.0	>3.0
Asymptomatic	Yes	0.139	>3.0	>3.0	>3.0
Asymptomatic	Yes	>3.0	>3.0	2.542	>3.0
Asymptomatic	Yes	0.365	>3.0	1.352	2.874
Hemophiliac	Yes	0.054	0.970	ND	0.304
Hemophiliac	Yes	0.296	0.997	ND	0.041
Hemophiliac	Yes	0.558	0.504	ND	0.171
Hemophiliac	Yes	0.174	1.194	ND	0.407
Hemophiliac	Yes	>3.0	>3.0	ND	1.458
Hemophiliac	Yes	0.026	1.201	ND	0.079
Hemophiliac	No	0.037	0.053	ND	0.029
Hemophiliac	No	0.062	0.055	ND	0.023
Hemophiliac	No	0.010	0.006	ND	0.019
High Risk	No	0.057	0.027	ND	0.095
High Risk	No	0.051	0.011	ND	0.053
Healthy Hetero.	No	0.003	0.019	0.006	0.106
Healthy Hetero.	No	0.079	0.038	0.050	0.009
Healthy Hetero.	No	0.042	0.111	0.013	0.060
Healthy Hetero.	No	0.053	0.040	0.061	0.093

BNSDOCID: <EP 0317804A2>

Table 3

Patient Diagnosis	Confirmed Seropos.	Microtiter Saliva IgA			
		p24	qp41	Peptide I	Peptide III
AIDS	Yes	0.076	0.099	0.938	1.480
AIDS	Yes	0.035	0.065	0.559	1.165
AIDS	Yes	0.077	0.007	0.232	0.440
AIDS	Yes	0.064	0.040	0.982	1.251
AIDS	Yes	0.096	0.151	1.010	1.604
AIDS	Yes	0.056	0.355	>3.0	>3.0
AIDS	Yes	0.002	0.175	0.449	1.120
AIDS	Yes	0.069	0.040	0.933	1.738
ARC	Yes	0.040	0.239	2.845	>3.0
ARC	Yes	0.094	0.172	2.036	2.836
Asymptomatic	Yes	0.144	0.078	0.840	1.054
Asymptomatic	Yes	0.419	0.110	>3.0	>3.0
Asymptomatic	Yes	0.288	0.773	1.635	>3.0
Asymptomatic	Yes	0.146	0.186	2.424	>3.0
Asymptomatic	Yes	0.634	0.678	1.544	2.988
Hemophiliac	Yes	0.045	0.020	ND	>3.0
Hemophiliac	Yes	0.003	0.008	ND	2.520
Hemophiliac	Yes	0.174	0.093	ND	1.835
Hemophiliac	Yes	0.140	0.094	ND	>3.0
Hemophiliac	Yes	0.280	0.435	ND	>3.0
Hemophiliac	Yes	0.072	0.063	ND	2.947
Hemophiliac	No	0.061	0.016	ND	0.103
Hemophiliac	No	0.003	0.046	ND	0.011
Hemophiliac	No	0.044	0.008	ND	0.038
High Risk	No	0.083	0.007	ND	0.055
High Risk	No	0.094	0.056	ND	0.103
Healthy Hetero.	No	0.002	0.064	0.073	0.054
Healthy Hetero.	No	0.054	0.013	0.007	0.098
Healthy Hetero.	No	0.016	0.017	0.084	0.008
Healthy Hetero.	No	0.042	0.004	0.067	0.051

Example 3

This example demonstrates a microparticle assay for detection of HIV antibodies utilizing Peptide III, recombinant DNA-derived p24 and recombinant DNA-derived gp41. One hundred microliters of amino-modified microparticles, 2.5% solids, 0.45 microns average diameter, commercially available from Polyscience, Warrington, Pennsylvania, were added to 1 ml of phosphate buffered saline (PBS) and 3.0 ml of sulfo-m-maleimido benzoyl-N-hydroxysuccinimide (Sulfo-MBS) (1.0 mg/ml in PBS). The solution was stirred for 1 hour at room temperature after which the microparticles were isolated by centrifugation at 5000 x g speed, washed twice with PBS and resuspended in 1 ml PBS. Thirty microliters of a solution of Peptide III (1 mg/ml in distilled water) were added to the resuspended microparticles and stirred for 2 hours at room temperature. The microparticles were isolated by centrifugation at 5000 x g speed, washed twice with PBS containing 0.05% Tween 20, and resuspended in PBS to yield a 0.125% solution. After resuspension in PBS, the particles were stored at 2-8° C for subsequent use in combination with p24 and gp41 coated microparticles in an assay for anti-HIV.

To make separate microparticle solutions of p24 coated microparticles and gp41 coated microparticles, one hundred microliters of amino-modified microparticles, 2.5% solids, 3.0 microns average diameter, commercially available from Polyscience, were added to 500 microliters of 50 mM N-methylmorpholine, pH

BNSDOCID: <EP 0317804A2>

7.5 and 300 microliters of 2-iminothiolane (10 milligrams per milliliter in ice-cold 0.1 M sodium bicarbonate) for each antigen. The solution was incubated for 1 hour at room temperature. Two hundred microliters of the p24 antigen and gp41 antigen (described in Example 2 except that concentrations were as follows: p24, 654 ug/ml; gp41, 100,000 units/ml) were each incubated with 200 microliters of Sulfo-MBS (3.75 ug/ml in PBS) for 1 hour at room temperature. Each activated antigen was then added to the microparticles and incubated overnight at room temperature. The microparticles were isolated by centrifugation, washed twice with PBS containing 0 05% Tween 20, and resuspended in PBS to yield a 0.125% solution. After resuspension in PBS, the particles were stored at 2-8° C for subsequent use in the microparticle assay for HIV antibodies described below.

Twenty microliters each of the antigen-coated microparticle solutions (Peptide III, p24 and gp41) were added dropwise to the center of a Whatman GF-D glass fiber filter arranged in a microparticle assay format. This assay format is described in more detail in copending U.S. Patent Application Serial No. 831,013, filed on Feb. 18, 1986, assigned to the same assignee as that of the present invention and incorporated by reference herein. Three hundred microliters of an overcoat solution (10% bovine serum albumin, 3% sucrose, 0.05% Tween 20 in PBS) were added and the unit incubated at 45° C for 90 minutes to dry the filter. It is to be noted that, in addition to the technique described in the foregoing examples, the antigens may be attached to the microparticles or other surfaces by a variety of methods, e.g., adsorption, use of specific antibodies, or the use of other various chemical activators.

A prefilter was situated above the unit, which contained the filter and the antigen-coated microparticles. Two hundred microliters of a 1:10 dilution of either serum or saliva in a sample diluent (10% bovine serum albumin, 2% Tween 20 in PBS) were added to the prefilter. After the sample was absorbed, 200 microliters of an antibody-enzyme conjugate comprising a mixture of goat anti-human IgG and IgA:alkaline phosphatase were added to the matrix through the prefilter. After absorption, the prefilter was removed and 1 milliliter of a detergent wash solution (1M guanadine hydrochloride, 1M NaCl, .05% Tween 20 in PBS) was added to the matrix to remove any excess antibody-enzyme conjugate. Then, 150 microliters of a chromogen indicator (bromo-chloro indolyl phosphate/nitro blue tetrazolium) were added to the matrix. After 2 minutes, 1 milliliter of the wash solution was added to the matrix. The matrix was checked visually. The appearance of a colored spot indicated that the specimen contained detectable levels of antibody to HIV. Samples tested by the foregoing procedure but not containing detectable levels of antibody to HIV produced no color in the matrix. The results are set forth in Tables 4 and 5.

BNSDOCID: <EP 0317804A2>

Table 4

Microparticle Assay				
Patient Diagnosis	Confirmed Seropos.	Serum		
		p24	qp41	p24,qp41,PIII
AIDS	Yes	Neg	Pos	Pos
AIDS	Yes	Neg	Pos	Pos
AIDS	Yes	Neg	Pos	Pos
AIDS	Yes	Neg	Pos	Pos
AIDS	Yes	Neg	Pos	Pos
AIDS	Yes	Neg	Pos	Pos
AIDS	Yes	Neg	Pos	Pos
AIDS	Yes	Neg	Pos	Pos
ARC	Yes	Neg	Pos	Pos
ARC	Yes	Neg	Pos	Pos
Asymptomatic	Yes	Pos	Pos	Pos
Asymptomatic	Yes	Pos	Pos	Pos
Asymptomatic	Yes	Pos	Pos	Pos
Asymptomatic	Yes	Pos	Pos	Pos
Asymptomatic	Yes	Pos	Pos	Pos
Hemophiliac	Yes	Pos	Pos	Pos
Hemophiliac	Yes	Pos	Pos	Pos
Hemophiliac	Yes	Pos	Pos	Pos
Hemophiliac	Yes	Pos	Pos	Pos
Hemophiliac	Yes	Pos	Pos	Pos
Hemophiliac	Yes	Pos	Pos	Pos
Hemophiliac	No	Neg	Neg	Neg
Hemophiliac	No	Neg	Neg	Neg
Hemophiliac	No	Neg	Neg	Neg
High Risk	No	Neg	Neg	Neg
High Risk	No	Neg	Neg	Neg
Healthy Hetero.	No	Neg	Neg	Neg
Healthy Hetero.	No	Neg	Neg	Neg
Healthy Hetero.	No	Neg	Neg	Neg
Healthy Hetero.	No	Neg	Neg	Neg

BNSDOCID: <EP 0317804A2>

Table 5

Microparticle Assay				
Patient Diagnosis	Confirmed Seropos.	Saliva		
		p24	qp41	p24,qp41,PIII
AIDS	Yes	Neg	Pos	Pos
AIDS	Yes	Neg	Neg	Pos
AIDS	Yes	Neg	Pos	Pos
AIDS	Yes	Neg	Neg	Pos
AIDS	Yes	Neg	Pos	Pos
AIDS	Yes	Neg	Pos	Pos
AIDS	Yes	Neg	Pos	Pos
AIDS	Yes	Neg	Pos	Pos
ARC	Yes	Neg	Pos	Pos
ARC	Yes	Neg	Pos	Pos
Asymptomatic	Yes	Pos	Pos	Pos
Asymptomatic	Yes	Pos	Pos	Pos
Asymptomatic	Yes	Pos	Pos	Pos
Asymptomatic	Yes	Pos	Pos	Pos
Asymptomatic	Yes	Pos	Pos	Pos
Hemophiliac	Yes	Neg	Pos	Pos
Hemophiliac	Yes	Pos	Pos	Pos
Hemophiliac	Yes	Pos	Pos	Pos
Hemophiliac	Yes	Neg	Pos	Pos
Hemophiliac	Yes	Pos	Pos	Pos
Hemophiliac	Yes	Neg	Pos	Pos
Hemophiliac	No	Neg	Neg	Neg
Hemophiliac	No	Neg	Neg	Neg
Hemophiliac	No	Neg	Neg	Neg
High Risk	No	Neg	Neg	Neg
High Risk	No	Neg	Neg	Neg
Healthy Hetero.	No	Neg	Neg	Neg
Healthy Hetero.	No	Neg	Neg	Neg
Healthy Hetero.	No	Neg	Neg	Neg
Healthy Hetero.	No	Neg	Neg	Neg

Example 4

Peptides I, II, III or IV, or any combination of these peptides with one another or with other HIV antigens, may be employed to produce antisera or as a vaccine.

Antisera is specifically produced by immunizing rabbits with injections of Peptides I or III according to the present invention as follows. The peptide is coupled to a carrier protein thyroglobulin by the following general procedure: To a solution of the selected peptide (2 mg) in 1 ml of either distilled water or dimethylformamide, is added 36 ul of a solution of 1-ethyl-3 (3-dimethylamino propyl) carbodiimide (Sigma Chemical Co., St. Louis, Missouri, 7.0 mg/ml H_2O) at 0° C. The mixture is stirred for 5-10 minutes at 0° C. Next, 0.5 ml of thyroglobulin solution (Sigma, 10 mg/ml in PBS) is added to this reaction mixture and stirred overnight at 0° C. Finally, the mixture is dialyzed against PBS buffer with three changes of the buffer.

New Zealand white rabbits were inoculated with 250 mg of the conjugated peptide mixed (1:1) with complete Freund's adjuvant. All subsequent boosts contained conjugated Peptide mixed 1:1 with incomplete Freund's adjuvant. Animals were bled two weeks after each boost. Bleeds were processed to yield polyclonal antibodies in the serum. Peptide antibodies so generated immunoprecipitated gp160 and gp120 from ^{35}S-methionine and ^{35}S-cysteine labelled cell lysates. These antibodies may, for example, be

BNSDOCID: <EP 0317804A2>

utilized as reagents in a diagnostic assay, for affinity purification of gp120 or gp160 antigens or as a passive vaccine for therapeutic or prophylactic applications.

An active vaccine solution according to the present invention may be prepared by suspending Peptides I, II, III or IV or a combination of these (or in combination with other HIV antigens) in an immunologically acceptable diluent, adjuvant or carrier. Initial and booster injections or oral delivery are used to confer immunity.

Example 5

Monoclonal antibodies according to the present invention may be produced by injecting mice with immunizing doses of Peptides I, II, III or IV or any combination of these with or without other epitopes of HIV. The peptide of interest is coupled to a carrier protein before injection as described in Example 4. The mouse spleens are then removed from the immunized animals and spleen cells are fused to myeloma cells (e.g. NS-1 cells) using polyethylene glycol. Hybridoma cells producing monoclonals are selected by screening in a suitable cell culture medium such as hypoxanthine aminoptern thymidine (HAT) medium. Monoclonal antibodies specific for HIV proteins may be isolated by affinity chromatography from media in which such hybridomas have been cultured.

The peptides of the invention have many advantages. First, they are noninfectious and therefore safer in diagnostic applications. Second, these peptides can be produced in large quantities at a low cost. Third, a diagnostic assay including these peptides alone, in combination with one another or in combination with other HIV epitopes is more sensitive and specific than previous HIV assays. Fourth, the peptides of the invention appear to have excellent application for saliva screening assays for HIV.

While specific examples have been given to illustrate the invention, it is to be understood that those skilled in the art will recognize variations which come within the scope of the invention as claimed. For example, the peptides of the invention may be used in conjunction with a number of HIV or other antigens or antibodies in diagnostic testing and in production of antibodies for vaccines or diagnostic assays.

Claims

1. A peptide selected from the group consisting of:
a) a Peptide of the formula:

(I) Y-Tyr-Lys-Tyr-Lys-Val-Ile-Lys-Ile-Glu-Pro-Leu-Gly-Ile Ala-Pro-Thr-Lys-Ala-Lys-Arg-Arg-Val-Val-Gln-Arg-Glu-Lys-Arg-X,

wherein Y corresponds to -H, an amino acid, peptide, protein or any linker, X is -OH, $-NH_2$, NR_1R_2 (wherein R is an alkyl group), a peptide, protein or any linker;

b) a peptide of the formula:

(II) Y-Arg-Asp-Asn-Trp-Arg-Ser-Glu-Leu-Tyr-Lys-Tyr-Lys-Val-Ile-Lys-Ile-Glu-Pro-Leu-Gly-Ile-Ala-Pro-Thr-Lys-Ala-Lys-Arg-Arg-Val-Val-Gln-Arg-Glu-Lys-Arg-X,

wherein Y corresponds to -H, an amino acid, peptide, protein or any linker, X is -OH, $-NH_2$, $-NR_1R_2$, (wherein R is an alkyl group), a peptide, protein or any linker;

c) a peptide of the formula:

(III) Y-Thr-Arg-Asp-Gly-Gly-Asn-Ser-Asn-Glu-Ser-Glu-Ile-Phe-Arg-Pro-Gly-Gly-Gly-Asp-Met-Arg-Asp-Asn-Trp-Arg-Ser-Glu-Leu-Tyr-Lys-Tyr-Lys-Val-Val-Lys-Ile-Glu-Pro-Leu-Gly-Val-Ala-Pro-Thr-Lys-Ala-Lys-Arg-Arg-Val-Val-Gln-Arg-Glu-Lys-Arg-X,

wherein Y corresponds to -H, an amino acid, peptide, protein or any linker, X is -OH, $-NH_2$, $-NR_1R_2$ - (wherein R is an alkyl group), a peptide, protein or any linker;

d) a peptide of the formula:

(IV) Y-Arg-Ser-Glu-Leu-Tyr-Lys-Tyr-Lys-Val-Val-Lys-Ile-Glu-Pro-Leu-Gly-Val-Ala-Pro-Thr-Lys-Ala-Lys-Arg-

BNSDOCID: <EP 0317804A2>

Arg-Val-Val-Gln-Arg-Glu-Lys-Arg-Ala-Val-Gly-Ile-Gly-Ala-Leu-Phe-Leu-Gly-Phe-Leu-Gly-Ala-Ala-Gly-Ser-Thr-X,

wherein Y corresponds to -H, an amino acid, peptide, protein or any linker, X is -OH, $-NH_2$, $-NR_1R_2$ - (wherein R is an alkyl group), a peptide, protein or any linker.

2. A peptide selected from the group consisting of Peptides I, II, III and IV and substitution, deletion and addition analogs thereof.

3. The peptide of claim 2 wherein said peptide is made in combination with other epitopes of HIV as a fusion protein.

4. The peptide of claim 2 in combination with an HIV antigen selected from the group consisting of HIV p24, gp41, gp120 and combinations thereof.

5. An immunoassay for detection of HIV antibodies in a biological sample comprising;

a) coating a solid phase with a peptide selected from the group consisting of Peptides I, II, III and IV;

b) incubating the solid phase with the biological sample;

c) incubating the solid phase with an anti-human Ig labeled with a detectable label; and

d) detecting the label to determine the presence of anti-HIV in the sample.

6. The immunoassay of claim 5 wherein the solid phase is further coated in step (a) with an HIV antigen selected from the group consisting of p24, gp41 and gp120 and combinations thereof.

7. The immunoassay of claim 5 wherein the solid phase has properties independently selected from one or more of the following:

a) the solid phase in step (a) is coated with the peptide of claim 3;

b) the anti-human Ig is selected from the group consisting of anti-human IgG, IgA, IgM and mixtures thereof;

c) the detectable label is selected from the group consisting of enzymes, radioisotopes and fluorescent molecules.

8. An immunoassay for detection of HIV antibodies in human saliva sample comprising:

a) coating a solid phase with a peptide selected from the group consisting of Peptides I, II, III and IV;

b) incubating the solid phase with the saliva sample;

c) incubating the solid phase with an anti-human Ig labeled with a detectable label; and

d) detecting the label to determine the presence of anti-HIV in the saliva sample.

9.The immunoassay of claim 8 wherein the solid phase has properties independently selected from one or more of the following:

a) the solid phase is further coated in step (a) with an HIV antigen selected from the group consisting of p24, gp41 and gp120 and combinations thereof;

b) the peptide is Peptide III and the HIV antigens are p24 and gp41;

c) the anti-human Ig is selected from the group consisting of anti-human IgG, IgA, IgM and mixtures thereof.

d) the detectable label is selected from the group consisting of enzymes, radioisotopes and fluorescent molecules.

10. An HIV antibody prepared by immunizing an animal with a peptide selected from the group consisting of peptides I, II, III, IV and mixtures thereof in a suitable adjuvant and obtaining antibodies from the sera of the animal.

11. The HIV antibody of claim 10 wherein the animal is immunized with the peptide in combination with at least one other HIV antigen.

12. A monoclonal antibody to HIV prepared by injecting an animal with an immunizing dose of a peptide selected from the group consisting of Peptides I, II, III, IV and mixtures thereof, removing the spleen from the animal, fusing the spleen with myeloma cells, culturing the fused cells in a suitable culture medium, and collecting HIV antibodies from said culture.

13. The monoclonal antibody of claim 12 wherein the animal is immunized with the peptide in combination with at least one other HIV antigen.

14. An HIV vaccine prepared by suspending a peptide selected from the group consisting of Peptides I, II, III, IV and mixtures thereof in a suitable pharmaceutically acceptable carrier.

BNSDOCID: <EP 0317804A2>

15. The HIV vaccine of claim 14 wherein the peptide is further combined with at least one other HIV antigen.

BNSDOCID: <EP 0317804A2>

(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) Publication number: **0 317 804 A3**

(12) EUROPEAN PATENT APPLICATION

(21) Application number: **88118299.2**

(22) Date of filing: **03.11.88**

(51) Int. Cl.[5]: **C07K 7/10, G01N 33/68, A61K 37/02, A61K 39/21, G01N 33/569, C12P 21/08**

(30) Priority: **24.11.87 US 124801**

(43) Date of publication of application:
31.05.89 Bulletin 89/22

(84) Designated Contracting States:
AT BE CH DE ES FR GB IT LI NL

(88) Date of deferred publication of the search report:
12.09.90 Bulletin 90/37

(71) Applicant: **ABBOTT LABORATORIES**
Abbott Park Illinois 60064(US)

(72) Inventor: **Sarin, Virender K.**
516 Fairlawn Avenue
Libertyville Illinois 60048(US)
Inventor: **Knigge, Kevin M.**
1083 Lawson Blvd.
Gurnee Illinois 60031(US)

(74) Representative: **Modiano, Guido et al**
MODIANO, JOSIF, PISANTY & STAUB
Modiano & Associati Baaderstrasse 3
D-8000 München 5(DE)

(54) HIV peptides and methods for detection of HIV.

(57) Novel synthetic peptides which substantially replicate various sequences of gp160 envelope protein of the human immunodeficiency virus (HIV) are disclosed. Also disclosed are methods for detection of antibodies to HIV in biological samples using the gp160 peptides.

EP 0 317 804 A3

BNSDOCID: <EP 0317804A3>

European Patent Office

EUROPEAN SEARCH REPORT

Application Number

EP 88 11 8299

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.4)
X	WO-A-8 602 383 (INSTITUT PASTEUR) * Claims 8,14,15; pages 13,23-24 *	1-3,5-7,12-15	C 07 K 7/10 G 01 N 33/68 A 61 K 37/02 A 61 K 39/21 G 01 N 33/569 C 12 P 21/00
X	EP-A-0 212 532 (SYNTEX) * Claims 1,10,13,16,18,20,25 *	1-3,5-7,12-15	
X	EP-A-0 231 914 (F. HOFFMANN-LA ROCHE, THE GOVERNMENT OF THE UNITED STATES OF AMERICA) * Claims 1,7,11,14,15,30,32 *	1-3,5-7,12-15	

TECHNICAL FIELDS SEARCHED (Int. Cl.4)

A 61 K
C 07 K

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	10-05-1990	PEETERS J.C.

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

EPO FORM 1503 03.82 (P0401)

BNSDOCID: <EP 0317804A3>